MYCOTAXON

ISSN (print) 0093-4666 (online) 2154-8889 Mycotaxon, Ltd. ©2019

April–June 2019—Volume 134, pp. 241–251

https://doi.org/10.5248/134.241

Inocybe caroticolor from oak forests of Pakistan

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Abstract—Basidiomata of *Inocybe caroticolor* were collected from two different *Quercus* forests in Swat, KP, Pakistan. Based on morphology and molecular phylogenetic analyses of ITS and 28S rDNA regions, these basidiomata were found to represent *I. caroticolor* and placed within the *Inocybe viscata* group. Our *I. caroticolor* specimens are molecularly supported as conspecific with specimens from China and represent a first species record from Pakistan.

Key words—GenBank, Himalayan, *Inocybaceae*, LSU

Introduction

The genus *Inocybe* (Fr.) Fr. (*Inocybaceae*) is distributed worldwide and covers a wide geographical range from tropical to arctic-alpine regions (Favre 1955; Horak 1979, 1980, 1981, 1987; Kühner 1988; Buyck & Eyssartier 1999; Watling 2001; Matheny & al. 2003; Jacobsson 2008). As one of the larger ectomycorrhizal genera in *Agaricales, Inocybe* is an important component of ectomycorrhizal temperate and boreal forest communities in alpine and arctic habitats (Cripps & al. 2010, Kokkonen & Vauras 2012).

Between 70% and 80% of the species in *Inocybaceae* have been described in associations primarily with ectomycorrhizal plant families *Fagaceae*, *Pinaceae*, and *Salicaceae* (Kirk & al. 2008). *Quercus* (oak), an important genus in the *Fagaceae,* is also known as the 'king of trees' due to its vital ecological role. In Pakistan, oaks are among the dominant vascular plants of the Himalayas, ranging from the subtropical to the sub-alpine zones

(Shrestha 2003). Himalayan oaks are evergreen, mostly gregarious, medium- to large-sized trees distributed at elevations of 800–3800 m a.s.l. throughout the Himalayan region. According to Alamgir (2004), established oak forests cover approximately 16,800 ha, making *Quercus* an important genus in temperate regions in Pakistan. Swat lies in Himalaya and its mycoflora is poorly known.

More than 850 *Inocybe* species have been reported worldwide (Matheny & al. 2009, 2012; Kobayashi & Onishi 2010; Horak & al. 2015; Jabeen & al. 2016). This number is increasing; during 2015–18 newly described species of *Inocybaceae* included 100 from Australia (Matheny & Bougher 2017), 19 from New Zealand (Horak 2018), 17 from India (Latha & Manimohan 2017), and 13 from Thailand and Malaysia (Horak & al. 2015). Species are also regularly being described in northern and southern Europe (e.g., Kokkonen & Vauras 2012; Esteve-Raventós & al. 2015, 2016, 2017; Bizio & al. 2016; Franchi & al. 2016; Vauras & Larsson 2016). *Inocybe* has been widely explored throughout Asia in China (Fan & Bau 2013, 2014), India (Vrinda & al. 1996, 1997; Pradeep & Vrinda 2007, 2010; Latha & Manimohan 2015, 2016, 2017; Tibpromma & al. 2017), Japan (Kobayashi & Courtecuisse 1993, 2000; Kobayashi & Hongo 1993; Kobayashi 2009; Kobayashi & Onishi 2010), and the Himalayas (Horak 1981). Only a few studies of the genus, however, have been undertaken in Pakistan. Since Ahmad & al. (1997) published their first report of *Inocybe* from Pakistan, 29 *Inocybe* species have been reported from different parts of the country (Ilyas & al. 2013, Saba & al. 2015, Jabeen & al. 2016, Farooqi & al. 2017, Naseer & al. 2018, Ullah & al. 2018), of which four—*I. ahmadii* Farooqi & al., *I. kohistanensis* Jabeen & al., *I. pakistanensis* Z. Ullah & al., and *I. shawarensis* Naseer & Khalid—were described as new to science (Jabeen & al. 2016, Farooqi & al. 2017, Naseer & al. 2018, Ullah & al. 2018).

During 2014–16 we collected specimens on field research trips while studying fungal communities associated with the oak forests of Pakistan. At that time we encountered in Swat an *Inocybe* species previously unknown from Pakistan. Its bright red carrot color easily distinguished this fungus in the field, and in the laboratory, we identified it as *Inocybe caroticolor*. Here we present a taxonomic description of our new Pakistani collections with the results of our ITS1-5.8S-ITS2 and 28S rDNA sequence analyses phylogenetically placing this species in the *Inocybe viscata* group of *Inocybe* clade (Matheny & Bougher 2017).

Material & methods

Collection & morphological examination

Specimens were collected from two different locations from the moist temperate forest of Toa, Swat as described in Naseer & al. (2017) and Shawar Valley as described in Naseer & al. (2018). These forests are dominated by *Quercus incana* Roxb. nom. illeg. [= *Q. oblongata* D. Don] and to a lesser extent *Q. dilatata* Royle. Gross morphological characters were recorded in the field and photographed using a Nikon D70S digital camera. Basidiomata were wrapped in aluminium foil and kept separately in a collection box to avoid mixing or crushing. Specimens were dried, sealed in plastic bag, and deposited in Lahore Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH). Colors were designated by mColorMeter. For anatomical examination, tissues from lamellae, pileipellis and stipitipellis were mounted on glass slides and observed in Phloxine (1%), Melzer's reagent, and KOH (5%) for pigmented hyphae and walls of cystidia using a Meiji Techno MX4300H microscope. Dimensions were measured for basidiospores, basidia and other elements from basidiomata under the light microscope equipped with a camera lucida.

Molecular phylogenetic analyses

DNA was extracted from lamellae of basidiomata following a modified CTAB method (Bruns 1995). The ITS region was amplified using primer pairs ITS1F/ITS4 (White & al. 1990) and the nrDNA 28S region using primer pairs LR0R/LR5 (Vilgalys & Hester 1990). Amplification parameters proceeded with an initial denaturation step at 94°C for 4 min, followed by 34 cycles of 94°C for 40 sec, 55°C (ITS) or 52°C (28S) for 40 sec, and 72°C for 1 min, and a final elongation step at 72°C for 8 min. The PCR products were purified, and Macrogen (South Korea) conducted bidirectional sequencing. The consensus sequences were subjected to BLAST, and compared with available GenBank sequences. The newly generated sequences were aligned with the closest matching NCBI sequences using MUSCLE v3.6 (Edgar 2004) and manually adjusted where necessary. ML tree generation and bootstrap analyses were performed for combined ITS and 28S data set on CIPRES Gateway (Miller & al. 2010). A maximum likelihood (ML) tree was inferred using RAxML-HPC2 v 8.1.11 (Stamatakis 2014) with a GTR + gamma model of nucleotide substitution. One thousand bootstrap iterations were performed with rapid bootstrapping. Significant support was considered to be ≥70%. Sequences generated in this study were submitted to GenBank.

Taxonomy

Inocybe caroticolor T. Bau & Y.G. Fan, Mycotaxon 123: 170 (2013) Figs 1, 2

Basidiomata small. Pileus 12–32 mm diam., bright red (2.4YR 3.1/9.3) in centre becoming yellowish (0.5Y 6.3/7) with patches of reddish orange; conical when young, then plano-convex, with an obtuse umbo, slightly rimulose, surface dry with appressed squamules over disc, radially fibrillose,

Fig. 1. *Inocybe caroticolor*. Basidiomata.
A, B. LAH35266; C, D. LAH35268; E–G. LAH35269.
Scale bars: A, B, E = 0.7 cm; C, D, F = 0.33 cm; G = 0.5 cm

rimose, margins inrolled (young) to incurved (mature). Lamellae light yellow (4.6Y 8.9/4.2) when young to yellowish orange (2.6YR 2.5/5.4) when mature, numbering 45–60, regularly arranged, adnexed, slightly close to crowded, 2–3 mm deep, edges even, smooth; lamellulae ¼ length of lamellae, single-tiered and alternating with lamellae. Stipe 32 × 4 mm, central, cylindrical, with subbulbous to non-marginate bulbous base, fibrillose, pruinose entire length, densely so at apex, longitudinally striate; yellow (4Y 6.2/6.5) in the middle region, and reddish orange (2.4YR 3.1/9.3) at apex, basal tomentum white.

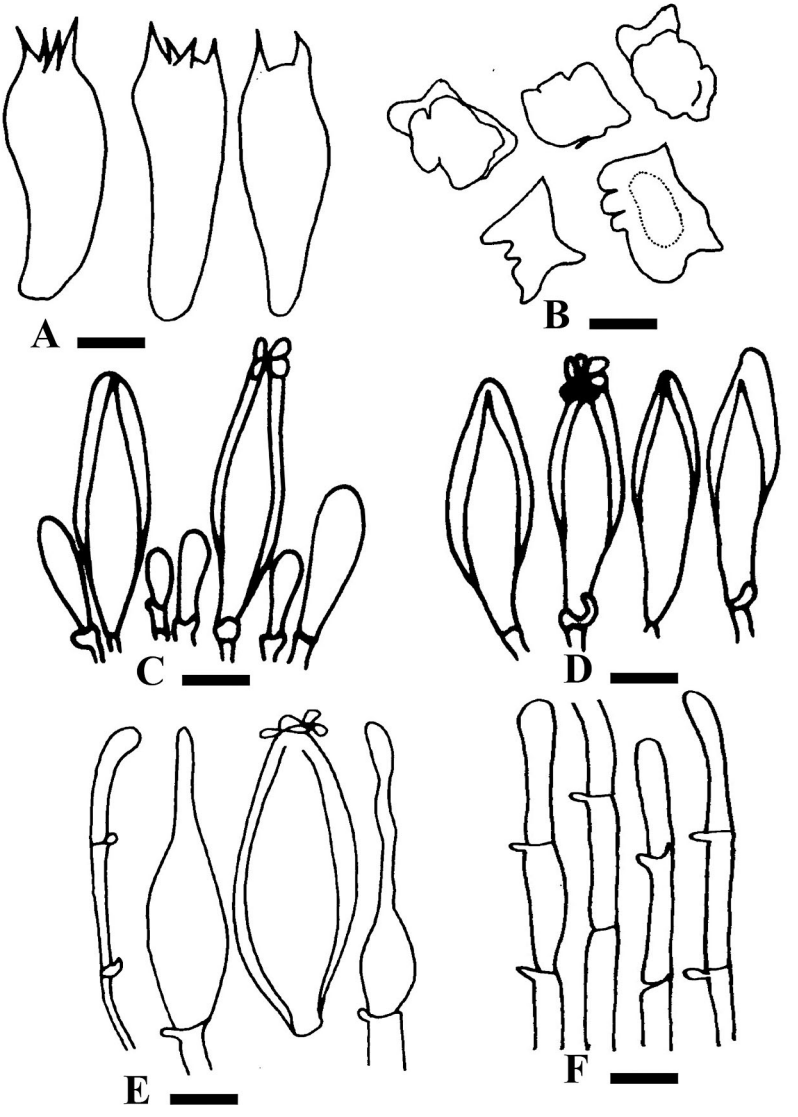

Fig. 2. *Inocybe caroticolor* (LAH35266). A. Basidia; B. Basidiospores; C. Cheilocystidia; D. Pleurocystidia; E. Caulocystidia with paracystidia; F. Pileipellis. Scale bars: A = 7.90 µm; B = 4.39 µm; C = 9.54 µm; D, E = 14 µm; F = 6.7 µm.

Basidiospores [n/b/p = 60/3/3], (6.3–)6.5–9.3(–9.5) × (4.5–)4.7–6.0 (–6.1) µm, mean = 7.5 × 5.2 µm, Q = (1.43–)1.47–1.58(–1.61), pale brown in 5% KOH, weakly to strongly nodulose. Basidia 25–36 × 5–8 µm, clavate, 4-spored, with yellowish pigments. Pleurocystidia 47–63 × 12–15 µm, fusiform, with crystalliferous apex, yellow in 5% KOH, thick walled, obtuse base. Cheilocystidia 48–62 × 12–15 µm, fusiform, some cylindrical. Caulocystidia 49–75 × 9–13 µm, variably shaped, abundant, thin-walled. Pileipellis 0.2–6.5 µm, a cutis of cylindrical hyphae, smooth, pigmented, thin-walled. Stipitipellis 2–4.5 µm, a cutis composed of regularly arranged hyphae, filamentous, smooth, thin- to moderately thick-walled. Clamp connections present.

Material examined: , Toa, 2100 m a.s.l, on soil under *Quercus oblongata*, 15 July 2015, Arooj Naseer, Abdul Rehman Niazi & Abdul Nasir Khalid AST47 (LAH35266; GenBank MH473148, MH536985); , Shawar Valley, 2100 m a.s.l, solitary on soil under *Q. dilatata* and *Q. oblongata* 14 July 2014, Arooj Naseer & Abdul Nasir Khalid ASSW11 (LAH35268; GenBank MH473144, MH536984); 15 Aug 2015, Arooj Naseer & Abdul Nasir Khalid ASS6 (LAH35267; GenBank MH473145, MH536986); 25 August 2014, Arooj Naseer & Abdul Nasir Khalid ASSW29 (LAH35269; GenBank MH473146, MH536983).

Molecular phylogenetic characterization

Sequencing of the ITS PCR products from *Inocybe caroticolor* specimens yielded 580–745 base pairs. NCBI BLAST searches of consensus sequences from four collections showed these sequences as 99% identical with *Inocybe caroticolor* (JX025772, JX025773 and JX025774) from China (100% query coverage; 0.0 E value). These sequences also showed a 93% identity with *Inocybe* sp. TR198-03 (KP636865) from Papua New Guinea.

Sequencing of the 28S PCR products yielded 867–980 base pairs. Consensus sequences of the 835 base pairs BLAST searched at NCBI showed 97% identity with *Inocybe* sp. REH7418 (JN974931) from an oak forest in Costa Rica and *Inocybe* sp.TR132-05 (KP171061) and *Inocybe* sp. TR88-06 (JN974992) from *Castanopsis* forests in Papua New Guinea with 100% query cover and 0.0 E value.

Sequences representing the *Inocybe viscata* group (*Inocybe* clade; Matheny & Bougher 2017), Indian species in a related clade (Latha & Manimohan 2017), and *Auritella dolichocystis* as outgroup were selected for preparation of the ITS/28S-based phylogram. Our newly generated sequences clustered with sequences from China with strong bootstrap value (Fig 3).

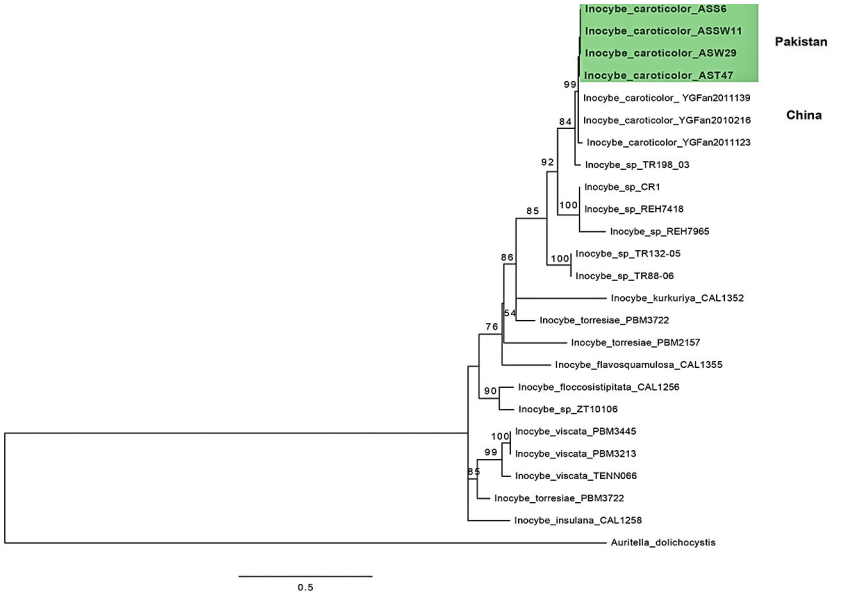

FIG. 3. ML phylogram of *Inocybe caroticolor* in the *I. viscata* group based on combined ITS and 28S nrDNA as generated with RAxML with 1000 bootstrap iterations. The letters in bold refer to sequences generated in this study, and sequences from Pakistan are highlighted in green.

Discussion

Inocybe caroticolor is characterized by the bright reddish carrot color of the basidiomata, nodulose basidiospores, and thick-walled hymenial cystidia. It can be easily recognized in the field by its bright red color, distinct aromatic odor, and entirely pruinose stipe. ITS sequence analysis clustered sequences from our Pakistani collections phylogenetically with those reported from China by Fan & Bau (2013), while the 28S phylogeny supported separation of the sequences from Pakistan in a separate clade from the sequences from *Inocybe* sp. TR88.06, *Inocybe* sp. TR132.05, and *Inocybe* sp. TR198.03 as observed in the ITS-based phylogram. However, as no other 28S-based sequences were present in GenBank, they were not included in a 28S-based phylogram.

The Pakistani collection of *I. caroticolor* differs somewhat morphologically from Chinese collection, which Fan & Bau (2013) described with a smaller

pileus and brighter red carrot color. In China, *I. caroticolor* has been found in association with *Quercus variabilis* Blume except for one collection found under *Pinus yunnanensis* Franch. (Fan & Bau 2013). During this study, all four collections from Pakistan were associated with *Q. oblongata* and *Q. dilatata* forests, indicating a strong preference for *Quercus*. This hypothesis is strengthened by the presence of other oak-specific species in the same clade (Matheny & Bougher 2017).

Our collections represent first record of *Inocybe caroticolor* for Pakistan. This species also exhibits a strong attraction for different oak species. In Pakistan, oak forests are being cut extensively for burning fuel and thus subject to decline. The presence of this unusual species may help develop an awareness of the need to conserve, establish, and reclaim these oak forests.

Acknowledgements

We are sincerely grateful to Dr. Brandon Matheny (University of Tennessee, Knoxville, USA) for his phylogenetic analyses and comments on an earlier version of this paper. We are thankful to Dr. Ditte Bandini (Wiesenbach, Germany) and Dr. Sana Jabeen (University of Education, Lahore, Pakistan) for their helpful comments to improve this manuscript.

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